



Attribution–NonCommercial–NoDerivs 2.0 KOREA

You are free to :

- **Share** — copy and redistribute the material in any medium or format

Under the following terms :



Attribution — You must give [appropriate credit](#), provide a link to the license, and [indicate if changes were made](#). You may do so in any reasonable manner, but not in any way that suggests the licensor endorses you or your use.




NonCommercial — You may not use the material for [commercial purposes](#).



NoDerivs — If you [remix, transform, or build upon](#) the material, you may not distribute the modified material.

You do not have to comply with the license for elements of the material in the public domain or where your use is permitted by an applicable exception or limitation.

This is a human-readable summary of (and not a substitute for) the [license](#).

[Disclaimer](#) 

약학석사학위논문

**Target identification research on the  
adiponectin-secreting activity of kojyl  
cinnamate derivatives in human  
mesenchymal stem cells**

사람의 중간엽줄기세포에서 아디포넥틴 분비촉진  
효과를 나타내는 코질신나메이트 유도체들의 타겟  
규명 연구

2017 년 8 월

서울대학교 약학대학원  
약학과 천연물과학전공  
김 세 언

## ABSTRACT

# **Target identification research on the adiponectin-secreting activity of kojyl cinnamate derivatives in human mesenchymal stem cells**

Sae On Kim

Department of Pharmacy

The Graduate School of Pharmacy

Seoul National University

Human mesenchymal stem cells (hMSCs) possess the capacity for multi-lineage differentiation. Under the appropriate conditions, hMSCs can undergo cell fate decision to commit to adipogenic, osteogenic, chondrogenic, or myogenic lineages. In this regard, the adipogenesis of human adipose tissue-derived mesenchymal stem cells (hAT-MSCs) can be used as a model for phenotype-based screening of anti-aging skin compounds with lipofilling effects.

The kojyl cinnamate derivative, Seletinoid G, has been clinically proven to have anti-aging effects in human skin. However, the efficacy in the phenotype assay was not sufficient to perform molecular target identification research. In this study, we

developed novel kojyl cinnamate derivatives for improved adiponectin promoting activity in hAT-MSCs. Through target deconvolution, we found that the adiponectin-secreting activities of kojyl cinnamates were correlated with PPAR $\gamma$  and PPAR $\alpha$ , but not PPAR $\delta$  activation. In a further investigation, we found that kojyl cinnamates with high binding affinities towards PPAR $\gamma$  and PPAR $\alpha$  significantly increased the expressions of cholesterol and fatty acid synthesizing enzymes in both hAT-MSCs and primary normal human keratinocytes (NHKs). Our findings suggest that kojyl cinnamate derivatives with PPAR $\gamma$  and PPAR $\alpha$  dual agonism have the potential to repair altered skin barrier function and improve aged-related decrease of subcutaneous adipose tissue.

**keywords :** Human adipose tissue-derived mesenchymal stem cells, Phenotype-based screening, Target deconvolution, Adipogenesis, Adiponectin, Peroxisome proliferator-activated receptor

***Student number :*** 2015-22389

# CONTENTS

<b>ABSTRACT .....</b>	<b>1</b>
<b>TABLE OF CONTENTS .....</b>	<b>3</b>
<b>LIST OF FIGURES .....</b>	<b>5</b>
<b>LIST OF TABLES .....</b>	<b>7</b>
<b>I. Introduction.....</b>	<b>8</b>
<b>II. Materials and Methods .....</b>	<b>11</b>
1. Cell culture and differentiation.....	11
2. Oil Red O and Hematoxylin staining .....	12
3. Enzyme-linked immunosorbent assay (ELISA) .....	12
4. Nuclear receptor (NR) assays .....	12
5. Total RNA isolation and quantitative real-time PCR (Q-RT-PCR).....	13
6. Statistical analysis .....	14
<b>III. Result.....</b>	<b>15</b>
1. Target identification of Seletinoid G. ....	15
2. Kojyl cinnamate derivatives promote adiponectin-secreting activity in hAT-MSCs during adipogenesis .....	19
3. Target deconvolution of kojyl cinnamate derivatives.....	24
4. Kojyl cinnamate derivatives modulate cholesterol and free fatty acid synthesis in aged skin models. ....	28
<b>IV. Discussion.....</b>	<b>43</b>

<b>V. Reference</b> .....	49
요약 (국문초록) .....	58

# LIST OF FIGURES

<b>Figure 1.</b> Chemical structures of retinoic acid, kojic acid, cinnamic acid, and Seletinoid G .....	16
<b>Figure 2.</b> Adiponectin promoting activity of Seletinoid G is independent of PPAR activation.....	17
<b>Figure 3.</b> Evaluation of the adiponectin-promoting activity of kojyl cinnamate derivatives during adipogenesis in hAT-MSCs .....	20
<b>Figure 4.</b> Kojyl cinnamate derivatives promote adiponectin production in a concentration-dependent manner .....	22
<b>Figure 5.</b> Nuclear binding activities of Seletinoid G and its related kojyl cinnamate derivatives .....	26
<b>Figure 6.</b> Correlation between adiponectin levels and PPAR binding affinities of Seletinoid G and its kojyl cinnamate derivatives .....	27
<b>Figure 7.</b> PPAR $\gamma$ and PPAR $\alpha$ binding activity of kojyl cinnamate derivatives.....	28
<b>Figure 8.</b> Role of PPARs in cutaneous biology .....	34
<b>Figure 9.</b> Kojyl cinnamate derivatives modulate cholesterol synthesis in differentiated hAT-MSCs .....	35
<b>Figure 10.</b> The effects PPAR $\alpha/\gamma$ dual agonists on lipogenesis in differentiated hAT-MSCs .....	37
<b>Figure 11.</b> Kojyl cinnamate derivatives regulate fatty acid synthesis in differentiated hAT-MSC.....	38

<b>Figure 12.</b> Transcriptional expression profile of ADIPOQ and FABP4 in differentiated hAT-MSCs.....	39
<b>Figure 13.</b> Kojyl cinnamate derivatives 5 and 7 modulate mevalonate pathway regulation in NHKs .....	40
<b>Figure 14.</b> Transcriptional expression profile of free fatty acid metabolism associated genes in NHKs .....	41
<b>Figure 15.</b> Schematic representation of the function of PPAR $\alpha$ / $\gamma$ dual agonists on the metabolic pathways leading to the synthesis of cholesterol and fatty acids in skin.....	42



# LIST OF TABLES

<b>Table 1.</b> PPAR $\gamma$ binding activity kojyl cinnamate derivatives .....	29
--	----

<b>Table 2.</b> PPAR $\alpha$ binding activity kojyl cinnamate derivatives .....	30
--	----

# I. Introduction

The phenotype-based approach to drug discovery can be used as an alternative to the conventional target-based method, and has the ultimate advantage of being able to evaluate candidate molecules at a more biologically and disease relevant context. However, the phenotypic method requires follow-up studies to elucidate the direct molecular targets responsible for the observed responses (1-2). Human mesenchymal stem cells (hMSCs) can be used to develop phenotype-based assays for various pathophysiological conditions. In particular, the adipogenesis of human adipose tissue-derived mesenchymal stem cells (hAT-MSCs) can be used as a model system to screen for active compounds that may improve age-related decrease of subcutaneous adipose tissue (3).

With increasing age, the adipose tissue undergoes significant changes in both composition and abundance. The age-dependent decline in adipogenesis and the subsequent regression of adipocyte lipid metabolism contribute to the progressive thinning and wrinkling of the skin (4-5). Adipogenesis involves a network of transcription factors that consists of peroxisome proliferator-activated receptor (PPAR) and CCAAT/enhancer binding proteins (C/EBPs), which collectively enhances the expressions of adipocyte specific genes (6-7). Adiponectin, which is expressed exclusively in adipose tissue, is an adipokine known to exhibit anti-inflammatory, pro-apoptotic, and insulin sensitizing properties (8-10). However, it is also involved in repairing altered connective tissue that results as of both intrinsic and extrinsic skin aging. For instance, adiponectin can improve age-

associated breakdown of extracellular matrix components, such as type 1 collagen and elastin (11-12). Adiponectin can be also up-regulated in response to pharmacological agents that modulate PPAR $\alpha$ , PPAR $\delta$ , and PPAR $\gamma$  activity, and examples include pioglitazone and rosiglitazone (13). Furthermore, its expression can be regulated by cyclin-dependent kinase 5 (CDK5), which phosphorylates the nuclear receptor PPAR $\gamma$  at serine 273 (14). In this regard, molecules with adiponectin-promoting activities during adipogenic differentiation can be investigated to determine whether they can interact with PPARs or CDK5.

In a previous study, we reported that the kojyl cinnamate ester derivative, 2-((3E)-4(2H,3H-benzo[3,4-d]1,3-dioxolan-5-yl)-2-oxo-but-3-enyloxy)-5-hydroxy-4H-pyran-4-one (Seletinoid G), repaired collagen and extracellular matrix (ECM) breakdown in aged skin by increasing the expressions of type 1 procollagen, tropoelastin, and fibrillin-2, while inhibiting the expression of interstitial metalloproteinases (MMPs), such as MMP-1 (15). We also found that Seletinoid G significantly promoted adiponectin production during adipogenesis in hAT-MSCs (16). However, the direct molecular targets responsible for the Seletinoid G-induced up-regulation of adiponectin production were not clearly identified due to its relatively weak potency on PPARs or CDKs (16). In this study, we synthesized novel kojyl cinnamate ester derivatives with more potent adiponectin-producing and -secreting activities to elucidate the molecular targets involved in the adipogenic differentiation of hAT-MSCs. Target deconvolution of the newly synthesized kojyl cinnamate ester derivatives demonstrated that the adiponectin-

producing and -secreting activities were associated with dual agonism on the nuclear receptors PPAR $\alpha$  and PPAR $\gamma$ .

## **II. Materials and Methods**

### **1. Cell culture and differentiation**

hAT-MSCs were purchased from Lonza, Inc. (Walkersville, MD) and maintained according to the manufacturer's instructions. hAT-MSCs were cultured in Dulbecco Modified Eagle's Medium (DMEM) containing low glucose (1 g/L) and supplemented with 10% fetal bovine serum (FBS), antibiotics, and Glutamax TM (Invitrogen, Carlsbad, CA). To promote adipocyte differentiation, the growth medium was replaced with DMEM containing high glucose (4.5 g/L) and supplemented with 10% FBS, 10 g/mL insulin, 0.5 M dexamethasone, and 0.5 mM 3-isobutyl-1-methylxanthine (IBMX) (IDX condition). Dexamethasone, insulin, IBMX, glibenclamide, troglitazone, Wy-14643, and aspirin were purchased from Sigma-Aldrich (St. Louis, MO, USA). hAT-MSCs were grown in the IDX condition and treated twice with either glibenclamide, troglitazone, Wy-14643, or compounds 1 to 12 for a total of 7 days.

Primary normal human keratinocytes (NHKs) from neonatal foreskin were purchased from Lonza (Basel, Switzerland) and cultured in KBM medium with KGM2 growth supplements consisting of insulin, human epidermal growth factor, bovine pituitary extract, hydrocortisone, epinephrine, transferrin, and gentamicin/amphotericin B (Sigma, St Louis, MO, USA). NHKs were treated with troglitazone, Wy-14643, or compounds 1 to 12 for a total of 24 h.

## **2. Oil Red O and Hematoxylin staining**

The level of adipocyte differentiation was assessed using Oil Red O stain from Sigma–Aldrich (St. Louis, MO, USA) as an indicator of intracellular lipid accumulation. Differentiated adipocytes were rinsed twice with phosphate-buffered saline (PBS), fixed with 10% formalin in PBS (pH 7.4) for 1 h, and then washed once with 60% isopropanol. Cultures were stained with 0.2% Oil Red O reagent for 10 min at room temperature and washed four times with H<sub>2</sub>O. To visualize the nucleus, the differentiated hBM-MSCs were counterstained with hematoxylin reagent from Sigma–Aldrich (St. Louis, MO, USA) for 1 min and then washed four times with H<sub>2</sub>O. The level of adipocyte differentiated was observed using an inverted phase-microscope.

## **3. Enzyme-linked immunosorbent assay (ELISA)**

The R&D Systems Quantikine™ immunoassay kit (Minneapolis, MN, USA) was used for quantitative measurement of adiponectin in cell culture supernatants. Cytokine concentrations were determined according to the manufacturer's instructions

## **4. Nuclear receptor (NR) assays**

Lanthascreen™'s time resolved fluorescence resonance energy transfer (TR-FRET) competitive binding assay kits (Invitrogen, Carlsbad, California, United States) were used to evaluate the PPAR $\alpha$ , PPAR $\delta$ , and PPAR $\gamma$  binding activities of chemical ligands. All assay measurements were performed according to the

manufacturer's instructions using CLARIOstar (BMG LABTECH, Ortenberg, Germany).

#### **4. Total RNA isolation and quantitative real-time PCR (Q-RT-PCR)**

Total RNA samples were isolated using Trizol's reagent (Invitogen, Carlsbad, CA, United States), followed by a purification step using the Qiagen RNeasy kit (Qiagen, Valencia, CA, United States). The concentration of RNA in each sample was determined spectrophotometrically at 260 nm. The integrity of each RNA sample was evaluated using Agilent 2100 BioAnalyzer (Agilent Technologies, Santa Clara, CA, USA). Total RNA from each sample was reversed transcribed to cDNA using the Superscript Reverse Transcriptase (RT) II Kit (Invitogen, Carlsbad, CA, United States). TaqMan Universal Master Mix II and Q-RT-PCR primer sets (Applied Biosystems, Foster City, CA, USA) were used to analyze cDNA samples for 3-hydroxy-3-methylglutaryl-CoA synthase 1 (HMGCS1, Hs00940429\_m1), 3-hydroxy-3-methylglutaryl-CoA synthase 2 (HMGCS2, Hs00985427\_m1), 3-hydroxy-3-methylglutaryl-CoA reductase (HMGCR, Hs00168352\_m1), Fatty acid synthase (FASN, Hs01005622\_m1), Fatty acid desaturase 1 (FADS1, Hs01096546\_m1), Acetyl-CoA carboxylase beta (ACACB, Hs01565914\_m1), Fatty acid desaturase 2 (FADS2, Hs00927433\_m1), Fatty acid binding protein 4 (FABP4, Hs01086177\_m1), Adiponectin (ADIPOQ, Hs00605917\_m1), and Acetyl-CoA acetyltransferase 2 (ACAT2, Hs00255067\_m1). Human glyceraldehyde-3-phosphate dehydrogenase (GAPDH, 4333764F) was used to

normalize sample variations. Q-RT-PCR was performed with an Applied Biosystems 7500 Real-Time PCR System (Applied Biosystems). Relative gene expression levels were quantified using equations from a mathematical model developed by Pfaffl.

## **6. Statistical analysis**

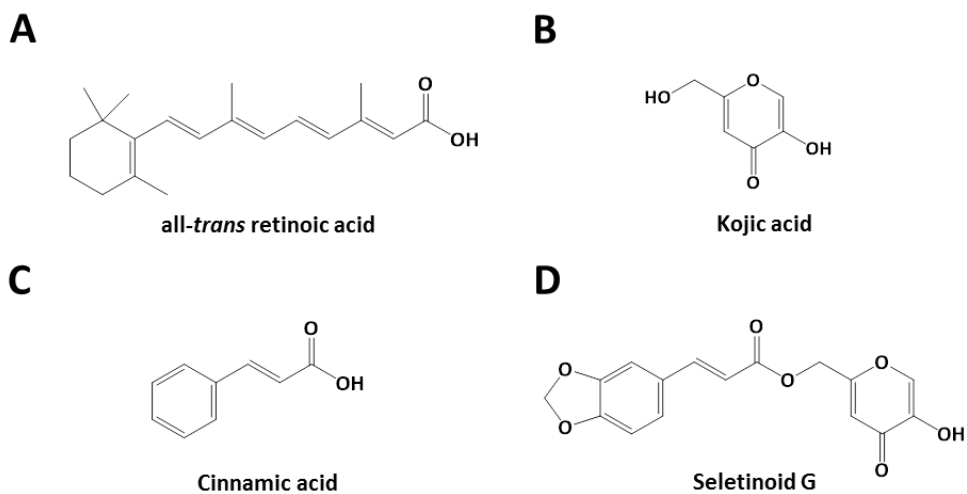
Experimental values are expressed as the mean  $\pm$  standard deviation. Statistical analysis was performed using one-way ANOVA. *P*-values of less than 0.05 and 0.01 were regarded as statistically significant.



### III. Results

#### 1. Target identification of Seletinoid G.

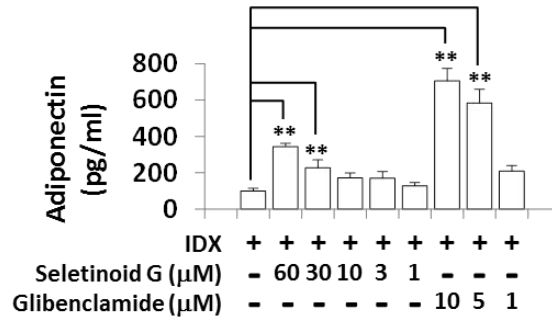
Seletinoid G is a synthetic retinoid with an  $\alpha$ ,  $\beta$ -unsaturated ketone group that was designed based on the structures of kojic acid and cinnamic acid. It was assumed that the  $\gamma$ -pyranone ring of the skin lightening-agent, kojic acid, would mimic the carboxylic acid moiety of the compound, retinoid acid, which exerts anti-aging activity in human skin (15). Furthermore, Seletinoid G was also designed with a cinnamate moiety based on the fact that the constituents of *Cinnamomum cassia* extracts can promote adipogenesis in hAT-MSCs (16). In our previous study, we found that the kojyl cinnamate derivative, Seletinoid G, can repair altered dermal connective tissue in aged and photoaged skin (15). In a follow up study, we found that Seletinoid G significantly increased adiponectin production at 30 $\mu$ M and 60 $\mu$ M in hAT-MSCs during adipogenesis (Figure 2A). Additionally, relative to the IDX control, we noticed that Seletinoid G increased both the number and size of lipid droplets in differentiated adipocytes (Figure 2B). However, the efficacy of Seletinoid G was too low to perform molecular target identification studies. When we evaluated the nuclear receptor binding activities of Seletinoid G to the PPAR isotypes, we found that it competitively displaced the selective ligands for PPAR $\gamma$ , PPAR $\alpha$ , and PPAR $\delta$  by less than 50% (Figure 1C). With this respect, we synthesized additional kojyl cinnamate derivatives for improved phenotypic activity in hAT-MSCs.



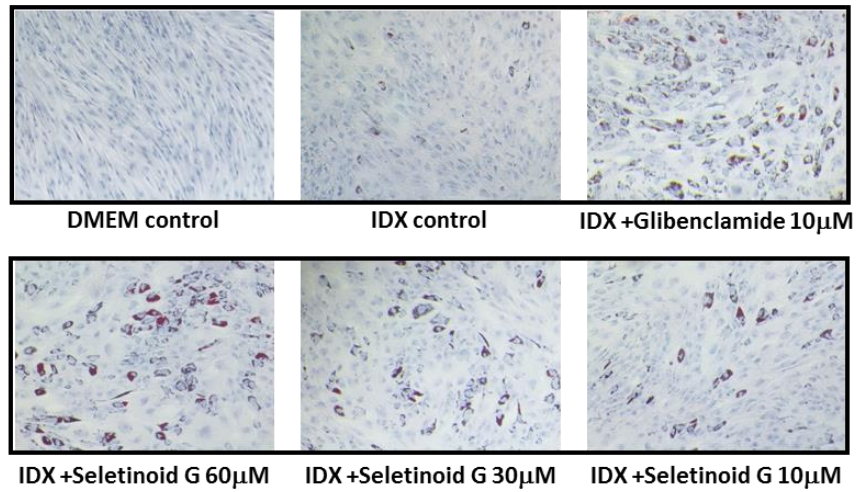
**Figure 1. Chemical structures of retinoic acid, kojic acid, cinnamic acid, and Seletinoid G**

Chemical structures of *all-trans* retinoic acid (A), kojic acid (B), cinnamic acid (C), and Seletinoid G (D) were drawn using ChemBioDraw Ultra<sup>®</sup> (Perkinelmer, Waltham, MA, USA).

**A**



**B**



**C**

Compound	PPAR $\gamma$ (% replacement)		PPAR $\alpha$ (% replacement)		PPAR $\delta$ (% replacement)	
	30 μM	10 μM	30 μM	10 μM	30 μM	10 μM
Seletinoid G	42.5 ± 7.7	16.8 ± 10.2	33.5 ± 2.7	24.2 ± 2.6	14.5 ± 4.4	0.9 ± 4.0

The positive controls for PPAR $\gamma$ , PPAR $\alpha$ , and PPAR $\delta$  were GW1929, GW7647, and GW0742, respectively. Values represent the mean binding activity ± SD (n=3).

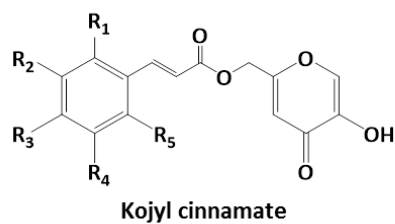
**Figure 2. Adiponectin promoting activity of Seletinoid G is independent of PPAR activation**

hAT-MSCs were grown under IDX conditions and/or co-treated with Seletinoid G at increasing concentrations. On the 7th day of culture, the cell supernatant was harvested and used to evaluate the levels of adiponectin secreted (A). Values represent mean adiponectin level  $\pm$  SD (n = 3). \*  $p \leq 0.05$  and \*\*  $p \leq 0.01$ . Oil red O staining was performed to determine the amount of lipid droplets accumulated over the 7 days of culturing in relation to the IDX control (B). DMEM was used as a negative control to indicate the absence of adipogenesis. Glibenclamide (10 $\mu$ M) was used as a positive control. TR-FRET NR competitive binding assays were performed to determine the binding activities of Seletinoid G to PPAR $\gamma$ , PPAR $\alpha$ , and PPAR $\delta$  (C).

## **2. Kojyl cinnamate derivatives promote adiponectin-secreting activity in hAT-MSCs during adipogenesis.**

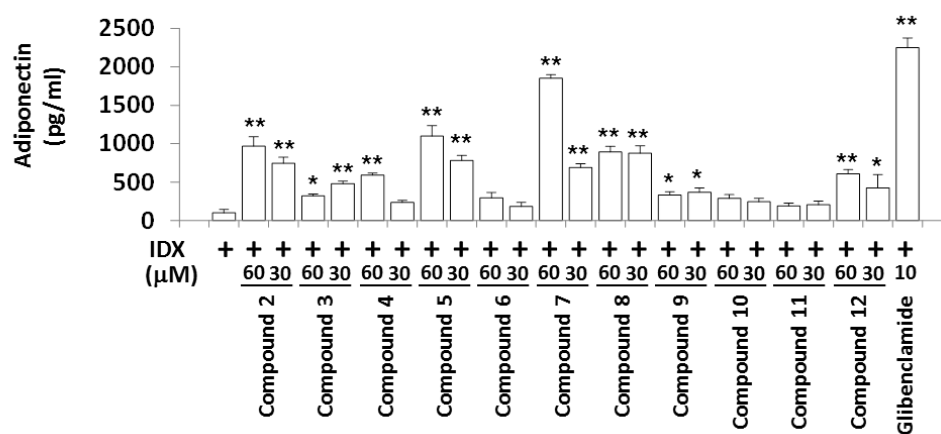
To determine whether the newly synthesized kojyl cinnamate derivatives promote adipogenesis in hAT-MSCs, compounds **2-12** were added to IDX supplemented medium and tested for adipogenic potential. As shown in Figure 3A, the addition of compounds **2**, **5**, **7**, and **8** to the IDX condition promoted adiponectin production in hAT-MSCs by 221%, 244%, 282%, 275%, respectively. In comparison to Sclerotinoid G, compounds **2**, **5**, **7**, and **8** exhibited potent adiponectin-promoting activities at both 30 $\mu$ M and 60 $\mu$ M (Figure 3B). In a follow-up study to examine a dose-dependence response, all four of the compounds showed maximal adiponectin production at non-cytotoxic concentrations of 60 $\mu$ M (Figure 4A). To further demonstrate the extent of adipogenesis in hAT-MSCs treated with kojyl cinnamate derivatives, Oil Red O and hematoxylin staining was used to assess the level of intracellular lipid accumulation. As anticipated, in comparison to the IDX control, compounds **2**, **5**, **7**, and **8** increased both the number and size of lipid droplets (Figure 4B).

**A**



Compound	Substituent					Adiponectin (pg/ml)
	R <sub>1</sub>	R <sub>2</sub>	R <sub>3</sub>	R <sub>4</sub>	R <sub>5</sub>	30 $\mu$ M
Seletinoid G	H	OCH <sub>2</sub> O(1,3dioxolyl)	H	H	H	154 $\pm$ 2**
2	OCH <sub>3</sub>	H	H	H	H	221 $\pm$ 30**
3	OCH <sub>3</sub>	OCH <sub>3</sub>	H	H	H	156 $\pm$ 4*
4	OCH <sub>3</sub>	OCH <sub>3</sub>	OCH <sub>3</sub>	H	H	137 $\pm$ 12*
5	OCH <sub>3</sub>	H	OCH <sub>3</sub>	H	H	244 $\pm$ 49**
6	OCH <sub>3</sub>	H	OCH <sub>3</sub>	OCH <sub>3</sub>	H	103 $\pm$ 30
7	OCH <sub>3</sub>	H	H	OCH <sub>3</sub>	OCH <sub>3</sub>	292 $\pm$ 81**
8	H	OCH <sub>3</sub>	H	H	H	275 $\pm$ 22**
9	H	OCH <sub>3</sub>	OCH <sub>3</sub>	H	H	120 $\pm$ 4
10	H	OCH <sub>3</sub>	OCH <sub>3</sub>	OCH <sub>3</sub>	H	137 $\pm$ 12
11	H	OCH <sub>3</sub>	H	OCH <sub>3</sub>	H	67 $\pm$ 14
12	H	H	OCH <sub>3</sub>	H	H	138 $\pm$ 112
IDX control						100 $\pm$ 15
Glibenclamide 10 $\mu$ M						722 $\pm$ 72**

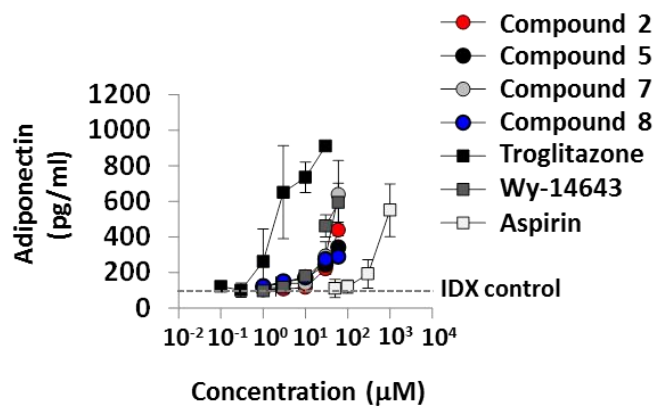
**B**



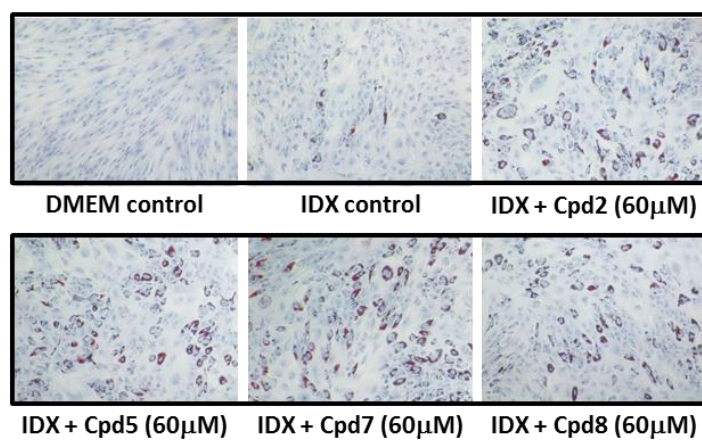
**Figure 3. Evaluation of the adiponectin-promoting activity of kojyl cinnamate derivatives during adipogenesis in hAT-MSCs**

The effects of kojyl cinnamate derivatives on the adiponectin-producing activities in hAT-MSCs during adipogenesis were evaluated using ELISA. hAT-MSCs grown under IDX supplemented medium and co-treated with compounds **2-12**. The levels of adiponectin in the cell culture supernatants were harvested 7 days after the initial treatment (A, B). Glibenclamide (10 $\mu$ M) was used as a positive control. Values represent mean  $\pm$  SD (n = 3). \*  $p \leq 0.05$  and \*\*  $p \leq 0.01$ .

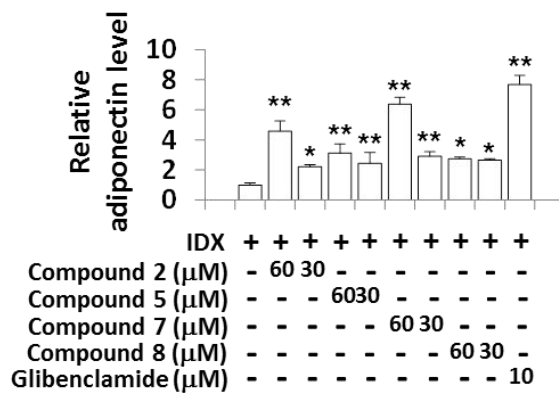
**A**



**B**



**C**





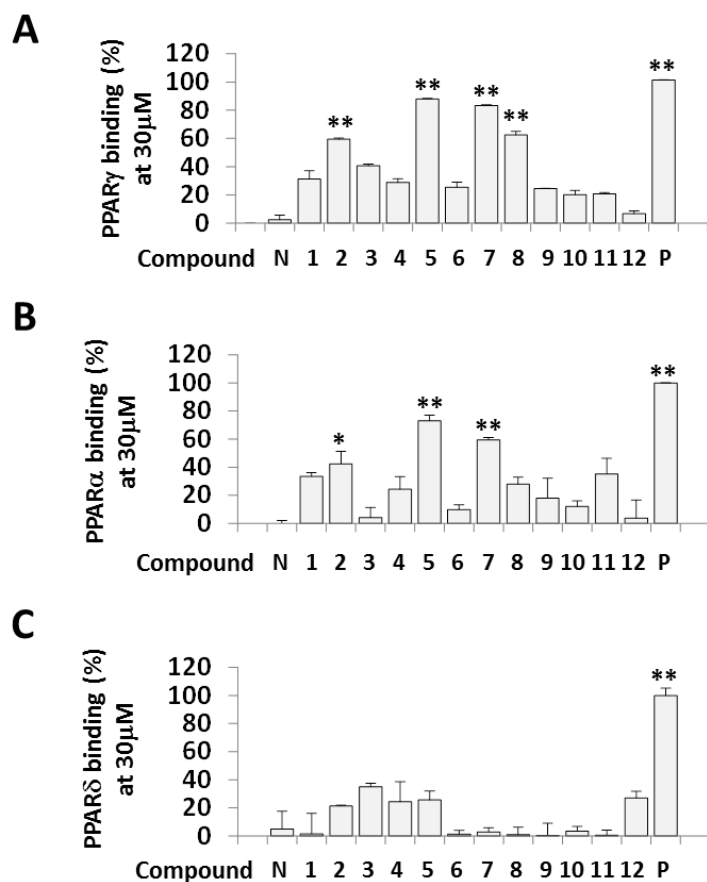
**Figure 4. Kojyl cinnamate derivatives promote adiponectin production in a concentration-dependent manner**

Concentration-dependent effects of kojyl cinnamate derivatives on adiponectin production during adipogenesis in hAT-MSCs. hAT-MSCs were treated with increasing concentrations of kojyl cinnamates in IDX supplemented medium. The cell culture supernatants were harvested on the 7<sup>th</sup> day in culture and used to measure the amount of adiponectin secreted. Troglitazone, Wy-14643, and Aspirin were used as positive controls (A). Values represent mean  $\pm$  SD (n=3). The levels of lipid accumulation of differentiated hAT-MSCs were qualitatively measured using Oil Red O staining. The extent of lipid accumulation was assessed relative to IDX control (B). The adiponectin levels of compounds **2**, **5**, **7**, and **8** at 30 $\mu$ M and 60 $\mu$ M were determined relative to the IDX control (C).

### 3. Target deconvolution of kojyl cinnamate derivatives

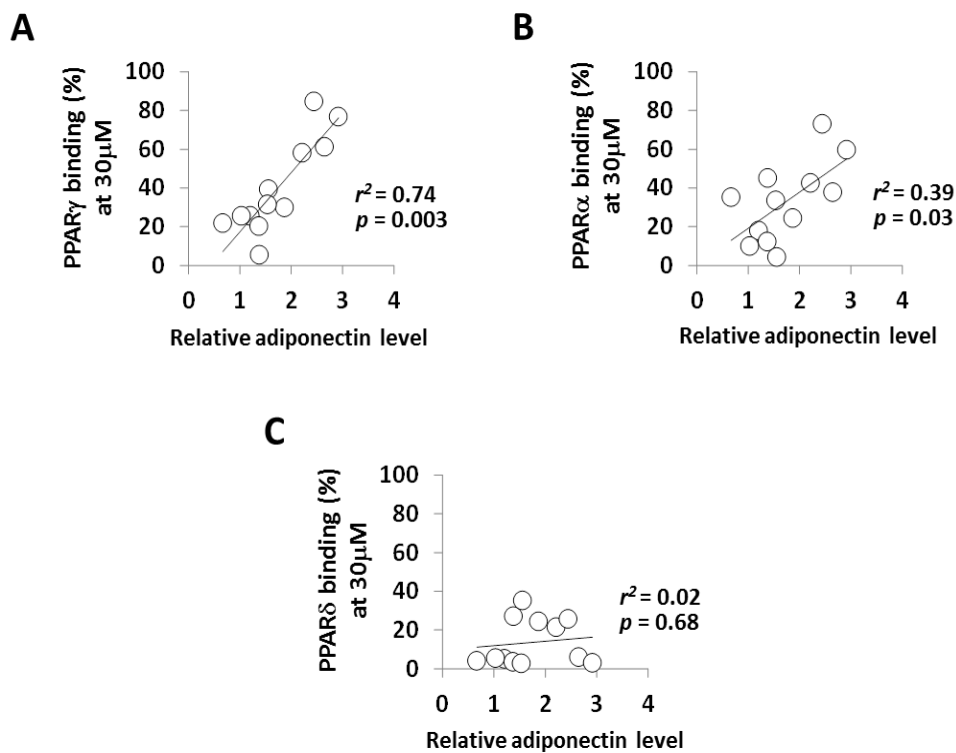
PPARs are involved in the process of adipocyte differentiation (17-19). Therefore, we speculated that the adiponectin-promoting potential of kojyl cinnamate derivatives in hAT-MSCs could be due to the activation of the PPAR signaling pathway. In this study, we evaluated the PPAR binding activities of kojyl cinnamates using TR-FRET NR competitive binding assays. Of the kojyl cinnamates, compounds **5** and **7** exhibited the highest binding affinities towards PPAR $\gamma$ , with overall binding activities of 84.6% and 76.8%, respectively (Figure 5A). Similarly, compounds **5** and **7** had the highest binding affinities towards PPAR $\alpha$ , and competitively displaced the fluorescently labeled PPAR $\alpha$  ligand by 73.0% and 59.6%, respectively (Figure 5B). In a further study, we found that there were significant correlations between the adipogenic potentials and the PPAR $\gamma$  and PPAR $\alpha$  binding activities of kojyl cinnamates. The correlation coefficient ( $r^2$ ) at 30  $\mu$ M was 0.74 ( $p < 0.003$ ) and 0.39 ( $p < 0.03$ ) for PPAR $\gamma$  and PPAR $\alpha$  respectively (Figure 6A, 6B). However, there was no significant correlation observed between the adiponectin-promoting activities and PPAR $\delta$  binding affinities of the newly synthesized kojyl cinnamates. The value of the correlation coefficient ( $r^2$ ) at 30  $\mu$ M was determined to be 0.02 ( $p < 0.68$ ) (Figure 6C). Next, we examined the dose–response relationship of the binding activities of compounds **5** and **7** to PPAR $\gamma$  and PPAR $\alpha$  subtypes. As anticipated, the kojyl cinnamate derivatives **5** and **7** displayed significant competitive binding activities in a concentration-dependent manner (Fig. 7A, 7B). The  $K_i$  values of compounds **5** and **7** were 6.7 and 7.0 for PPAR $\gamma$  and 8.0 and 9.5 for PPAR $\alpha$ , respectively (Table 1, 2). Consistent with the literature, the

agonists Troglitazone and Wy-14643 bound specifically to PPAR $\gamma$  and PPAR $\alpha$ , respectively (Figure 7A, 7B). The competitive and selective activation of PPAR $\gamma$  and PPAR $\alpha$  suggests that the binding activities of the two PPAR isotypes are positively correlated with the adipogenic potential of kojyl cinnamate esters during adipogenesis in hAT-MSCs.



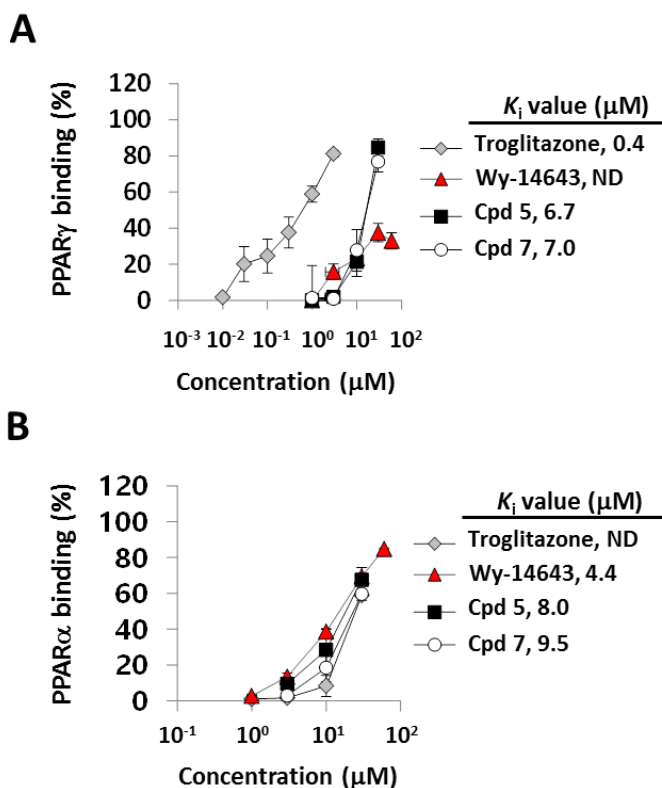
**Figure 5. Nuclear binding activities of Seletinoid G and its related kojyl cinnamate derivatives**

PPAR binding affinities of Seletinoid G and its kojyl cinnamate derivatives. TR-FRET competitive binding assays of compounds 1-12 were performed at 30 $\mu$ M as ligands of (A) PPAR $\gamma$ , (B) PPAR $\alpha$ , and (C) PPAR $\delta$ . The positive controls (P) were GW1929 for PPAR $\gamma$ , GW7647 for PPAR $\alpha$ , and GW0742 for PPAR $\delta$ . N stands for negative control. Values from the present study are expressed as mean  $\pm$  standard SD (n = 3) \* p < 0.05 and \*\* p < 0.01.



**Figure 6. Correlation between adiponectin levels and PPAR binding affinities of Sletinoid G and its kojyl cinnamate derivatives**

The correlation coefficients between the adiponectin levels and PPAR $\gamma$  (A), PPAR $\alpha$  (B), and PPAR $\delta$  (C) binding affinities at 30  $\mu$ M of kojyl cinnamates were calculated using Pearson's correlation coefficients ( $r^2$ ). Values represent mean  $\pm$  SD ( $n = 3$ ).



**Figure 7. PPAR $\gamma$  and PPAR $\alpha$  binding activity of kojyl cinnamate derivatives**

In order to determine the dose-dependent binding affinities of compounds 5 and 7 to PPAR $\gamma$  (A) and PPAR $\alpha$  (B) nuclear receptors, TR-FRET competitive binding assays were performed. The inhibition constant ( $K_i$ ) was calculated by applying the Cheng-Prusoff equation. The positive controls included Troglitazone for PPAR $\gamma$  and Wy-14643 for PPAR $\alpha$ . DMSO in buffer was used as a blank control. Values were expressed in terms of percentage compared to each positive control. Percentage values represent mean  $\pm$  SD (n = 3).

**Table 1. PPAR $\gamma$  binding activity of kojyl cinnamate derivatives**

Compound	PPAR $\gamma$ (% replacement)			
	30 $\mu$ M	10 $\mu$ M	3 $\mu$ M	$K_i$ <sup>a</sup>
Seletinoid G	31.4 $\pm$ 5.7	7.9 $\pm$ 3.3	0.5 $\pm$ 4.0	16.6 $\pm$ 2.0
Cpd2	59.5 $\pm$ 0.9**	11.4 $\pm$ 2.3	ND	ND
Cpd3	40.7 $\pm$ 1.1	6.5 $\pm$ 2.4	ND	ND
Cpd4	28.9 $\pm$ 2.6	8.1 $\pm$ 2.9	ND	ND
Cpd5	84.6 $\pm$ 0.4**	21.2 $\pm$ 8.0	2.0 $\pm$ 3.5	6.5 $\pm$ 0.25
Cpd6	25.5 $\pm$ 3.5	0.7 $\pm$ 1.5	ND	ND
Cpd7	76.8 $\pm$ 0.6**	27.8 $\pm$ 11.5	0.9 $\pm$ 2.5	7.1 $\pm$ 0.1
Cpd8	62.6 $\pm$ 2.3**	10.6 $\pm$ 2.0	ND	ND
Cpd9	24.6 $\pm$ 0.2	6.0 $\pm$ 2.0	ND	ND
Cpd10	20.3 $\pm$ 3.0	1.1 $\pm$ 3.4	ND	ND
Cpd11	20.8 $\pm$ 1.1	14.2 $\pm$ 0.6	ND	ND
Cpd12	6.8 $\pm$ 1.9	0.1 $\pm$ 0.7	ND	ND

<sup>a</sup> The  $K_i$  value for the PPAR $\gamma$  agonist Troglitazone was 0.3  $\pm$  0.14. The binding activity of the positive controls GW1929 (1 $\mu$ M) and Troglitazone (3 $\mu$ M) was determined to be 100.1% and 94.1%, respectively. Values represent the mean binding activity  $\pm$  SD (n=3). \* p <0.05 and \*\* p <0.01

**Table 2. PPAR $\alpha$  binding activity of kojyl cinnamate derivatives**

Compound	PPAR $\alpha$ (% replacement)			
	30 $\mu$ M	10 $\mu$ M	3 $\mu$ M	$K_i$ <sup>a</sup>
Seletinoid G	33.5 $\pm$ 2.7	8.4 $\pm$ 1.7	1.1 $\pm$ 2.3	16.4 $\pm$ 1.21
Cpd2	42.5 $\pm$ 8.7*	2.0 $\pm$ 1.0	ND	ND
Cpd3	4.3 $\pm$ 7.1	0.1 $\pm$ 15.9	ND	ND
Cpd4	24.3 $\pm$ 9.0	19.0 $\pm$ 17.4	ND	ND
Cpd5	73.0 $\pm$ 3.9**	20.1 $\pm$ 6.5	0.7 $\pm$ 0.5	8.0 $\pm$ 0.67
Cpd6	10.0 $\pm$ 3.3	0.9 $\pm$ 2.5	ND	ND
Cpd7	59.6 $\pm$ 1.7**	18.5 $\pm$ 2.6	2.9 $\pm$ 2.8	9.5 $\pm$ 0.22
Cpd8	37.8 $\pm$ 27.2	27.9 $\pm$ 5.1	ND	ND
Cpd9	17.9 $\pm$ 14.3	8.7 $\pm$ 19.1	ND	ND
Cpd10	12.2 $\pm$ 4.0	0.3 $\pm$ 1.8	ND	ND
Cpd11	35.2 $\pm$ 11.2	28.9 $\pm$ 3.9	ND	ND
Cpd12	45.1 $\pm$ 21.4	3.8 $\pm$ 12.9	ND	ND

<sup>a</sup>The  $K_i$  value for the PPAR $\alpha$  agonist Wy-14643 was 4.4  $\pm$  1.36. The binding activity of the positive controls GW7647 (1 $\mu$ M) and Wy-14643 (30 $\mu$ M) were determined to be 98.9% and 94.7%, respectively. Values represent the mean binding activity  $\pm$  SD (n=3). \* p <0.05 and \*\* p <0.01



#### **4. Kojyl cinnamate derivatives modulate cholesterol and free fatty acid synthesis in aged skin models**

Next, to investigate the role of PPAR activation on skin homeostasis, we analyzed the mRNA levels of key enzymes involved lipid metabolism. Ligands of PPAR $\gamma$  and PPAR $\alpha$  are important modulators of lipogenesis and help promote adipogenic differentiation (17, 20). Although there have been a lot of studies based on PPAR mono-agonists in cutaneous biology, the role of PPAR $\alpha/\gamma$  dual agonists in human skin models still remain to be identified. Aged skin is characterized by an overall decrease in lipid synthesis and is particularly associated with a profound decrease in cholesterol production (21). To investigate whether PPAR $\gamma$  and PPAR $\alpha$  dual activation can reverse altered cholesterol metabolism in aged skin, we evaluated the effects of kojyl cinnamate derivatives on the mRNA levels of the cholesterol metabolizing enzymes, 3-hydroxy-3-methylglutaryl-coenzyme A reductase (HMGCR), 3-hydroxy-3-methylglutaryl-coenzyme A synthase 1 (HMGCS1), and acetyl-coenzyme A acetyl transferase 2 (ACAT2) in differentiated hAT-MSCs. In this study, we found that compounds **5** and **7** significantly increased the mRNA levels of HMGCR and HMGCS1 at 24h ( $p < 0.01$ ) (Figure 9A-9C). We also noticed the mRNA expressions of fatty acid desaturase 1 and 2 (FADS1, FADS2), fatty acid synthase (FASN), acetyl CoA carboxylase B (ACACB) were markedly up-regulated by compounds **5** and **7** after 24h ( $p < 0.05$ ) (Figure 10-11). Our results suggest that the kojyl cinnamate derivatives **5** and **7** have the potential to increase cholesterol and fatty acid biosynthesis in human subcutaneous adipose tissue. Next,

to confirm that the activation of PPAR $\gamma$  and PPAR $\alpha$  by kojyl cinnamate derivatives promotes adipogenesis in hAT-MSCs, the mRNA levels of ADIPOQ and FABP4 were quantified. In this study, the expression of the PPAR downstream target genes, ADIPOQ and FABP4, were measured as markers of adipogenesis. As anticipated, the PPAR $\gamma$  agonist, Troglitazone, increased the transcription of adipogenic genes associated with mature adipocyte phenotype. In contrast, despite its lipid accumulating effect, the PPAR $\alpha$  agonist, Wy-14643, had no significant effect on the mRNA levels of ADIPOQ and FABP4 in hAT-MSCs. However, the combined treatment of the two mono-agonists synergistically up-regulated the transcription of genes involved in adipocyte differentiation. Similarly, compounds **5** and **7**, which exhibit PPAR $\gamma$  and PPAR $\alpha$  dual agonism, increased both ADIPOQ and FABP4 gene transcription ( $p < 0.05$ ,  $p < 0.01$ ) (Figure 12A, 12B).

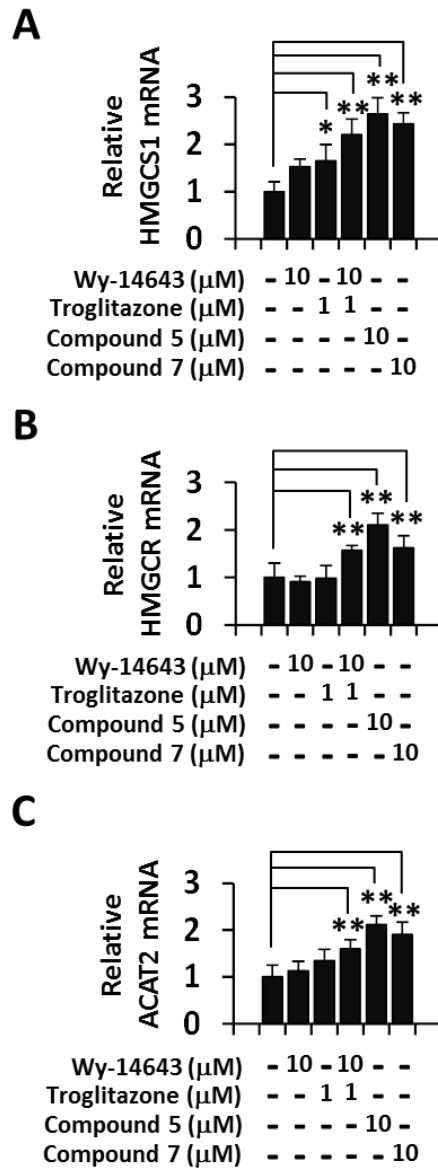
Aged skin displays an overall reduction in epidermal lipid synthesis, which results in abnormal barrier function (21). In order to investigate whether kojyl cinnamate derivatives can improve decreased skin barrier capacity, we quantified the mRNA levels of enzymes involved in cholesterol and free fatty acid metabolism. Similarly to the results observed in hAT-MSCs, compounds **5** and **7** significantly increased HMGCS1 and ACAT2 expressions in primary normal human keratinocytes (NHKs) at 24h ( $p < 0.05$ ) (Figure 13A, 13D). Although the kojyl cinnamate derivatives **5** and **7** increased HMGCR mRNA expression, it was not statistically significant (Figure 13C). Interestingly, we found that only the PPAR $\alpha$  agonist, Wy-14643, significantly up-regulated HMGCS2 expression ( $p < 0.01$ ) (Figure 13B). In this present study, we also found that compounds **5** and **7** significantly up-regulated the expressions of FADS1, FADS2, and FABP4 in NHKs after 24h ( $p < 0.05$ ) (Figure

14). Our results suggest that PPAR $\gamma$  and PPAR $\alpha$  dual agonists, such as compounds **5** and **7**, have the potential to repair epidermal barrier abnormality by increasing the rate of lipid synthesis in human skin (Figure 15).

	PPAR $\alpha$ agonist	PPAR $\gamma$ agonist	PPAR $\alpha/\gamma$ dual agonist
Epidermis	Increases lipid synthesis Promotes stratum corneum formation Increases lamellar body formation Promotes epidermal differentiation (filaggrin, involucrin, loricrin) Promotes barrier recovery Exerts anti-inflammatory effects (TNF- $\alpha$ , IL-1 $\alpha$ , IL-1 $\beta$ , IL-6, IL-8, PIGF, etc.)	Increases lipid synthesis Increases lamellar body formation Promotes epidermal differentiation (filaggrin, involucrin, loricrin) Decreases epidermal proliferation Promotes barrier recovery Exerts anti-inflammatory effects (TNF- $\alpha$ , IL-1 $\alpha$ , IL-1 $\beta$ , IL-6, IL-8, PIGF, etc.)	?
Dermis	Inhibits MMP expression Increases ECM synthesis Promotes procollagen synthesis Induces catalase expression and ROS scavenging	Promotes wound healing Increases insulin sensitivity Stimulates adipogenic differentiation of cultured fibroblasts	?
Subcutis	Promotes lipogenesis Induces adiponectin receptor expression	Promotes adipocyte differentiation Increases insulin sensitivity Promotes lipogenesis Induces adipokine secretion (adiponectin, leptin, etc.)	?

**Figure 8. Role of PPARs in cutaneous biology**

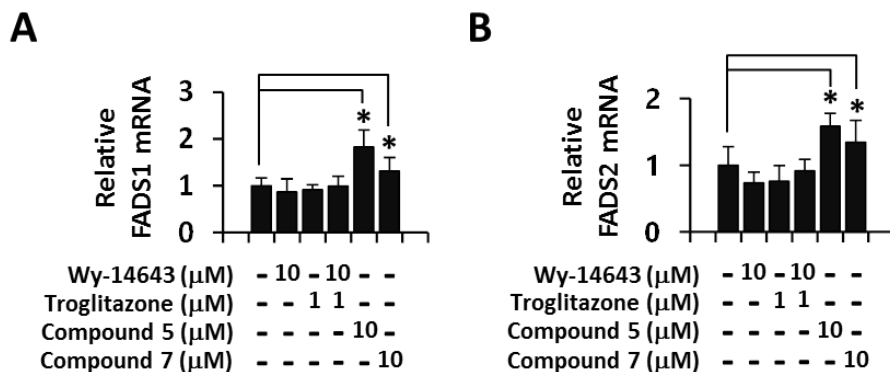
The known functions of PPAR $\alpha$  and PPAR $\gamma$  in skin homeostasis. PPAR  $\alpha$  and  $\gamma$  isotypes modulate lipogenesis in both human epidermal and subcutaneous tissues. The effects of PPAR $\alpha/\gamma$  dual agonists on human aged skin models remain to be identified.



**Figure 9. Kojyl cinnamate derivatives modulate cholesterol synthesis in differentiated hAT-MSCs**

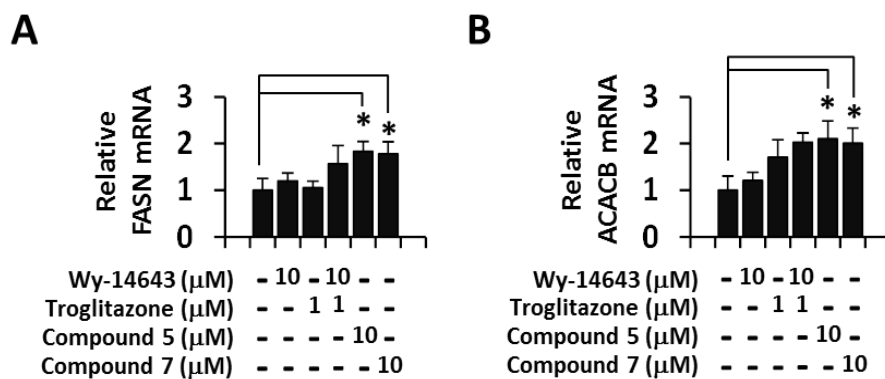
Differentiated hAT-MSCs were treated with Wy-14643, Troglitazone, compound 5 or compound 7. 24h after treatment, total RNA was extracted and Q-RT-PCR was

performed for HMGCS1, HMGCR, and ACAT2 (A-C). GAPDH was used as an internal control for Q-RT-PCR standardization. The positive controls included Wy-14643 for PPAR $\alpha$ , Troglitazone for PPAR $\gamma$ , and the co-treatment of Wy-14643 and Troglitazone for PPAR $\alpha/\gamma$ . Values from the present study are expressed as means  $\pm$  SD (n = 3). \* p <0.05 and \*\* p <0.01.



**Figure 10. The effects of PPAR $\alpha$ / $\gamma$  dual agonists on lipogenesis in differentiated hAT-MSCs**

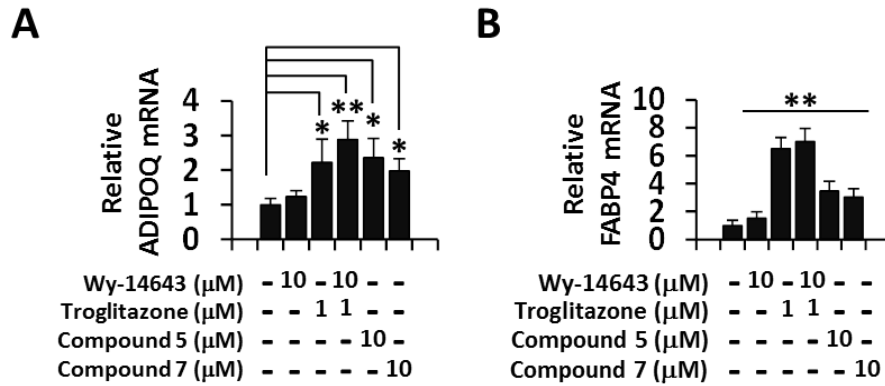
The effects of kojyl cinnamate esters on the expression of genes associated with lipogenesis in differentiated hAT-MSCs. 24h after compound treatment, total RNA was extracted and Q-RT-PCR was performed for FADS1 and FADS2 (A, B). GAPDH was used as an internal control for Q-RT-PCR standardization. The positive controls for PPAR $\alpha$  and PPAR $\gamma$  were Wy-14643 and Troglitazone, respectively. Wy-14643 and Troglitazone co-treatment was used as a positive control for PPAR $\alpha$ / $\gamma$ . Values from the present study are expressed as means  $\pm$  SD (n = 3). \* p < 0.05 and \*\* p < 0.01.



**Figure 11. Kojyl cinnamate derivatives regulate fatty acid synthesis in differentiated hAT-MSC**

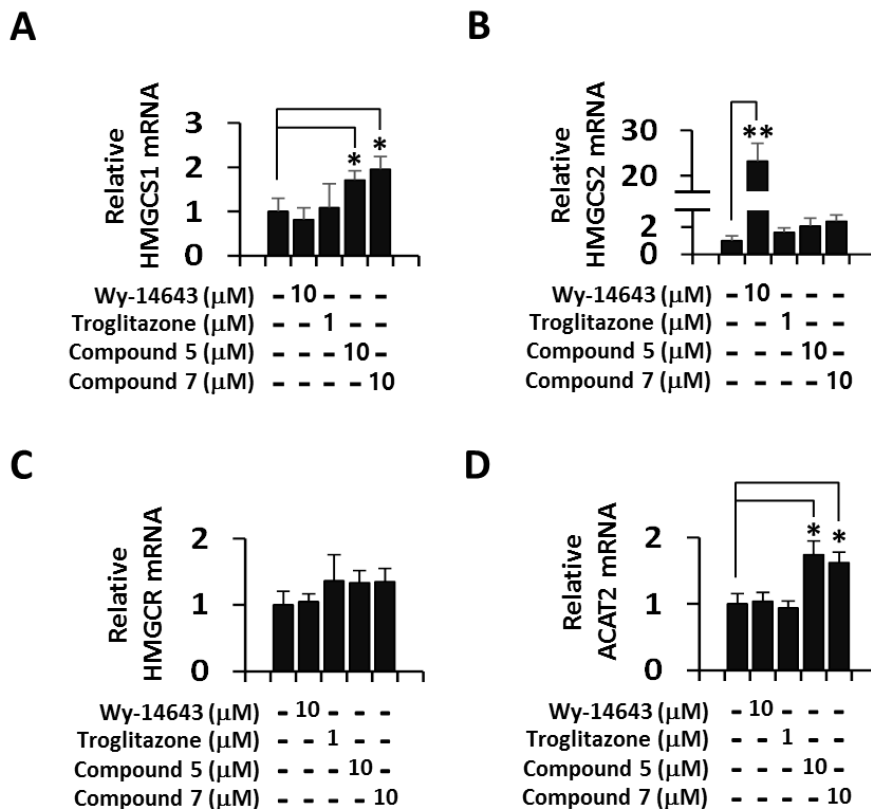
IDX-induced differentiated hAT-MSCs were treated with Wy-14643, Troglitazone, or compounds 5 and 7. 24h in culture, total RNA was extracted and Q-RT-PCR was performed for FASN (A) and ACACB (B). GAPDH was used as an internal control for Q-RT-PCR standardization. Results are the mean  $\pm$  SD ( $n = 3$ ). \*  $p < 0.05$  and \*\*  $p < 0.01$ .





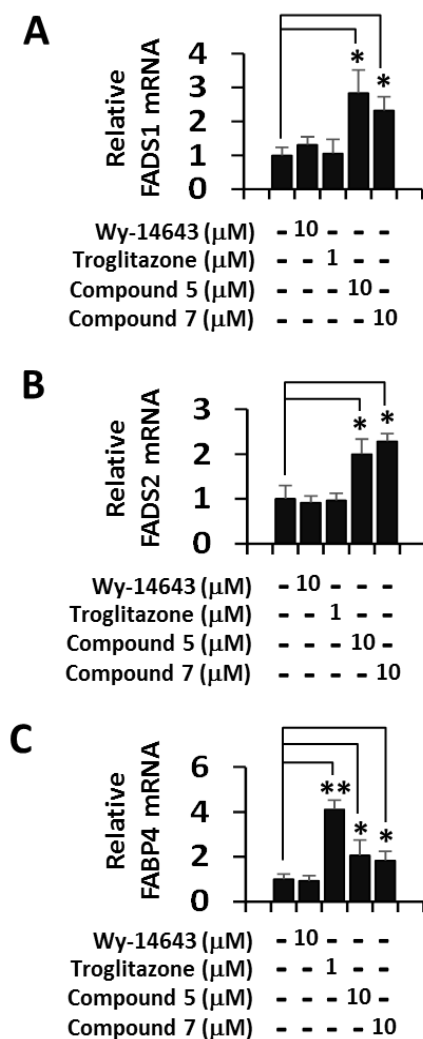
**Figure 12. Transcriptional expression profile of ADIPOQ and FABP4 in differentiated hAT-MSCs**

IDX-induced differentiated hAT-MSCs were treated with Wy-14643, Troglitazone, or compounds 5 and 7. 24h in culture, total RNA was extracted and Q-RT-PCR was performed for ADIPOQ (A) and FABP4 (B). GAPDH was used as an internal control for Q-RT-PCR standardization. Results are the mean  $\pm$  SD (n = 3). \* p < 0.05 and \*\* p < 0.01.



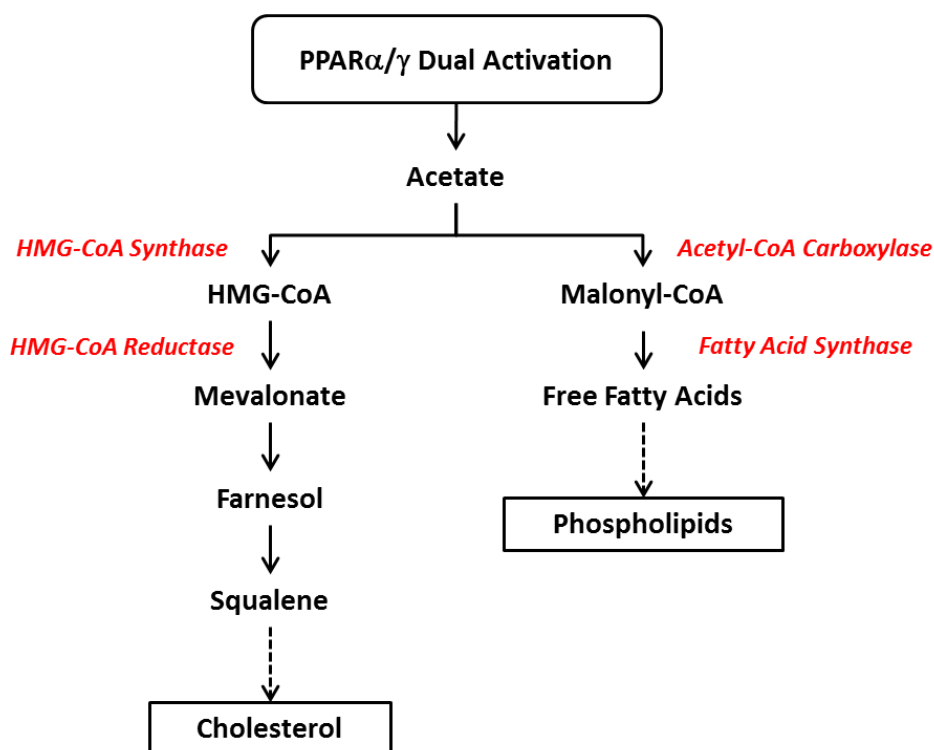
**Figure 13. Kojyl cinnamate derivatives 5 and 7 modulate mevalonate pathway regulation in NHKs**

NHKs were treated with Wy-14643, Troglitazone, and compound 5 or 7 in KGM medium. 24h after treatment, total RNA was extracted and Q-RT-PCR was performed for HMGCS1, HMGCS2, HMGCR, and ACAT2 (A-D). GAPDH was used as an internal control for Q-RT-PCR standardization. Values from the present study are expressed as means  $\pm$  SD (n = 3). \* p < 0.05 and \*\* p < 0.01.



**Figure 14. Transcriptional expression profile of free fatty acid metabolism associated genes in NHKs**

NHKs were treated with Wy-14643, Troglitazone, compound 5 or compound 7. At 24h in cell culture, total RNA was extracted and Q-RT-PCR was performed for FADS1, FADS2, and FABP4 (A-C). GAPDH was used as an internal control for Q-RT-PCR standardization. Values from the present study are expressed as means  $\pm$  SD (n = 3). \* p < 0.05 and \*\* p < 0.01.



**Figure 15. Schematic representation of the function of PPARα/γ dual agonists on the metabolic pathways leading to the synthesis of cholesterol and fatty acids in skin**

PPARα/γ dual agonists, such as kojyl cinnamate esters **5** and **7**, promote cholesterol and free fatty acid synthesis in the human skin by increasing the expression of lipogenic enzymes, such as HMG-CoA synthase, HMG-CoA reductase, acetyl-CoA carboxylase, and fatty acid synthase. PPARα and PPARγ dual activators can act as modulators of skin barrier homeostasis and may accelerate barrier recovery in aged skin.

## IV. Discussion

The aim of this study was to investigate the mechanism by which kojyl cinnamate derivatives exert anti-aging potential in human skin. In our prior study, we found that the kojyl cinnamate derivative, Seletinoid G, promoted ECM synthesis and adiponectin production in cell-based assays (16-17). When we performed a target identification study to evaluate whether Seletinoid G transactivated nuclear receptors involved in the regulation of adipogenesis, we found that there were no significant activities detected (17). In order to elucidate the molecular targets involved in the adiponectin-promoting activity of kojyl cinnamate derivatives in hAT-MSCs, we synthesized additional compounds with kojyl cinnamate pharmacores for improved efficacy in phenotype-based assays. In this study, we demonstrated that the compounds **5** and **7** promote adiponectin production in IDX-induced differentiated adipocytes. Subcutaneous adipose tissue-derived adiponectin can stimulate the production of major ECM components in the dermis and can reduce age-related decrease of skin elasticity and volume (9, 22-23). Previous studies have also demonstrated that adiponectin from subcutaneous adipocytes can decrease collagen breakdown by inhibiting MMP activity, and conversely enhance procollagen synthesis by dermal fibroblasts (11, 22-23). Moreover, adiponectin can also regulate multiple activities in the skin by mediating endocrine, paracrine or autocrine mechanisms (24-27). Adiponectin secreted from subcutaneous adipose tissues may act in a paracrine manner to regulate anti-aging mechanisms in the dermal and epidermal tissues of the skin. With this respect, it can be suggested that

compounds **5** and **7** may have the ability to indirectly increase ECM production in human skin.

In this study, we hypothesized that the PPAR signaling pathway may be involved in the adiponectin-secreting activity of kojyl cinnamate derivatives in hAT-MSCs during adipogenesis. PPARs are ligand-activated transcription factors that belong to the family of nuclear receptors, and consist of three isotypes ( $\alpha$ ,  $\delta$ , and  $\gamma$ ), which are expressed in the human skin (28-29). These nuclear receptors have multiple roles in skin homeostasis, and equally participate in the formation of the skin barrier (29-31). Moreover, the cross-talk between the PPAR isotypes and their transcriptional network are known to be involved in the modulation of adipocyte differentiation (7, 17, 32). Although kojyl cinnamate esters have been shown to promote adiponectin production in hAT-MSCs during adipogenic differentiation, the pharmacological mechanisms behind their phenotypic changes are not fully understood (17). In the present study, we demonstrated that the activation of PPAR $\alpha$  and PPAR $\gamma$  by kojyl cinnamate esters is positively correlated with the levels of secreted adiponectin. Furthermore, we revealed that the adiponectin-promoting activity of kojyl cinnamates does not involve the activation of PPAR $\delta$ . Previous studies have demonstrated that PPAR $\alpha$  activators, such as fibrates, can induce the expression of adiponectin and its associated receptors in adipose tissues (33-35). It has also been reported that PPAR $\gamma$  can act as a master regulator of adipocyte differentiation, and that its activation by selective ligands, such as thiazolidinediones (TZDs), can up-regulate the expression and secretion of adiponectin (36-38). Consistent with these reports, our findings demonstrate that

PPAR $\alpha$  and PPAR $\gamma$  dual-agonists, such as compounds **5** and **7**, are important modulators of adipocyte differentiation, and that their activation stimulates both the expression and secretion of adiponectin in subcutaneous adipose tissue.

More importantly, we also demonstrated that PPAR $\alpha$  and PPAR $\gamma$  activation increases lipogenesis in differentiated adipocytes. Phenotypic evaluation using Oil Red O staining revealed that the kojyl cinnamate esters, **5** and **7**, enhance both the number and size of lipid droplets in hAT-MSCs. In a further study, we found that PPAR $\alpha$  activation up-regulate the transcription of enzymes involved in cholesterol and fatty acid synthesis. Aged skin is characterized by the reduced ability of subcutaneous adipocytes to produce and accumulate essential lipids, and the inability to maintain adequate levels of lipids may contribute to the overall weakening and thinning of the skin (39-42). PPAR $\alpha$  activation by selective agonists, such as Wy-14643, can reverse age-related decrease of lipid synthesis, and knocking-out its expression in the skin can cause structural and functional abnormalities that delays barrier recovery (20, 43). According to past studies, PPAR $\alpha$  agonists can increase mRNA expressions of key enzymes involved in cholesterol, fatty acid, and ceramide synthesis (44-46). Consistent with these findings, we found that Wy-14643-induced PPAR $\alpha$  activation increases mRNA levels of mevalonate pathway associated genes, such as HMGCS1, HMGCR, and ACAT2. However, we noticed that there was a synergistic effect on lipid synthesis when both PPAR $\alpha$  and PPAR $\gamma$  were simultaneously activated. Relative to the PPAR $\alpha$  and PPAR $\gamma$  treatments alone, the co-treatment of the agonists Wy-14643 and Troglitazone enhanced the expressions of cholesterol synthesizing enzymes.

Similarly, we found that the PPAR $\alpha$ / $\gamma$  dual agonists, **5** and **7**, stimulate cholesterologenesis in hAT-MSCs. Cholesterol plays an important role in dermal ECM remodeling, and is known to mediate collagen degradation by MMPs. Low levels of cholesterol are associated with an increase in MMP-9 expression, and inducing cholesterol synthesis can abrogate ERK-JNK pathway dependent activation of AP-1 transcription factor, which is involved in the inhibition of procollagen synthesis (47-51). Overall, these findings suggest that PPAR $\alpha$ / $\gamma$  dual agonists, such as compounds **5** and **7**, may exhibit anti-aging potential in the skin by indirectly promoting ECM synthesis.

Likewise, to determine whether PPAR $\alpha$  and PPAR $\gamma$  dual activation enhances the activities of key regulators involved in fatty acid biosynthesis, we evaluated the mRNA levels of FASN and ACACB. Our findings indicate that relative to the PPAR $\alpha$  and PPAR $\gamma$  activations alone, the activation of the two PPAR isotypes promotes fatty acid synthesis in hAT-MSCs. The subcutaneous adipose tissue of chronologically and photo-aged skin is characterized by a marked decrease in fatty acid levels, and this can be reversed by stimulating the expression of lipid synthesizing enzymes (52-53). Additionally, it has been suggested that fatty acid desaturase (FADS) deficiency in aged skin may be associated with abnormal skin lipid composition, and may result in pathological conditions like atopic dermatitis and psoriasis (53-54). In this present study, we evaluated whether kojyl cinnamate derivatives with PPAR $\alpha$  and PPAR $\gamma$  agonism can up-regulate the activity of FADS, which are involved in modulating cutaneous lipogenesis. Our findings demonstrated that compounds **5** and **7** significantly increase FADS1 and FADS2



expression in differentiated hAT-MSCs. FADS are expressed in multiple tissues, including the skin, and are known to decrease in activity over age (54-55). Based on clinical studies of adipose tissue fatty acid composition, it has been indicated that reduced levels of FADS are associated with abnormal barrier function that results as of decreased levels of essential long-chain fatty acid derivatives in the permeability barrier (56-58). These findings demonstrate that compounds **5** and **7** may exhibit anti-aging potential in the skin by promoting FADS activity, which decreases over age. Taken together, our results suggest that PPAR $\alpha$  and PPAR $\gamma$  dual activation by kojyl cinnamate esters can improve age-dependent decline of adipogenesis and lipogenesis in subcutaneous adipose tissue.

Aside from their roles in hAT-MSCs, PPARs are known to be involved in both the differentiation and formation of the epidermal barrier (59-63). According to past studies, it has been demonstrated that PPAR $\alpha$  and PPAR $\gamma$  activators accelerate barrier recovery in skin constructs following acute barrier abrogation by stimulating epidermal lipid synthesis and lamellar body formation (20, 64-66). Impaired barrier function is heavily associated with the decreased incidence of epidermal lipid synthesis, and enhancing the rates of cholesterol, fatty acid and ceramide synthesis to maintain lipid ratios of 3:1:1, respectively, can accelerate barrier recovery (66-69). Chronologically and photo-aged skin displays an overall reduction in lipid content, and is particularly associated with a profound decrease in cholesterol levels (67, 70-75). Although there has been a lot of focus based on the effects of PPAR $\alpha$  and PPAR $\gamma$  mono-activators on the stimulation of human epidermal lipogenesis, there have been very few reports based on the roles of

PPAR $\alpha$ / $\gamma$  co-agonists. Here, we demonstrated that PPAR $\alpha$  and PPAR $\gamma$  dual activation by kojyl cinnamate derivatives **5** and **7** can increase the activities of rate-limiting enzymes involved in the synthesis of cholesterol in NHKs. Topical application of PPAR $\alpha$ , PPAR $\delta$ , and PPAR $\gamma$  selective activators, such as Wy-14643, GW1514, and ciglitazone, has been demonstrated to increase lipogenesis in both human and murine models of aged or barrier disrupted skin (20, 33, 46, 52). Consistent with these findings, we demonstrated that the PPAR isotypes  $\alpha$  and  $\gamma$  have the potential to improve altered barrier function in human skin by enhancing epidermal lipid synthesis. However, we found that relative to the Wy-14643 and Troglitazone treatments alone, the PPAR $\alpha$ / $\gamma$  dual agonists, **5** and **7**, synergistically enhanced cholesterol synthesizing enzymes, such as HMGCS1, HMGCR, and ACAT2. Moreover, similar to the observations made in differentiated hAT-MSCs, we found that the kojyl cinnamate derivatives increased the expression of enzymes involved in the formation and regulation of essential fatty acids. All together, these findings suggest that the dual activation of PPAR $\alpha$  and PPAR $\gamma$  by kojyl cinnamate esters can normalize altered epidermal cholesterol and fatty acid synthesis in aged skin models. However, the effects of kojyl cinnamate derivatives on ceramide synthesis remain to be elucidated. Further studies should focus on the underlying mechanisms behind PPAR $\alpha$  and PPAR $\gamma$  activation in the epidermal lipid synthesis and skin barrier function.

## V. Reference

1. Schenone M, et al. Target identification and mechanism of action in chemical biology and drug discovery (2013) *Nat Chem Biol* 9: 232-240.
2. Gujral TS, Peshkin L, Kirschner MW (2014) Exploiting polypharmacology for drug target deconvolution. *Proc Natl Acad Sci USA* 111(13): 5048-5053.
3. Noh M, et al. Interleukin-17A increases leptin production in human bone marrow mesenchymal stem cells (2010) *Biochem Pharmacol* 83: 661-670.
4. Cartwright MJ, Tchkonja T, Kirkland JL Aging in adipocytes: potential impact of inherent, depot-specific mechanisms (2007) *Exp Gerontol* 42: 463-471.
5. Kamel AF, et al. (2004) Age-dependent regulation of lipogenesis in human and rat adipocytes *J Clin Endocrinol Metab* 89(9): 4601-4606.
6. Cristancho AD, Lazar MA (2011) Forming functional fat: a growing understanding of adipocyte differentiation *Nat Rev Mol Cell Biol* 12: 722-734.
7. Rosen ED, MacDougald OA (2006) Adipocyte differentiation from the inside out *Nat Rev Mol Cell Biol* 7: 885-896.
8. Turer AT, Scherer PE (2012) Adiponectin: mechanistic insights and clinical implications *Diabetologia* 55: 2319-2326
9. Kim M, et al. (2016) Adiponectin suppresses UVB-induced premature senescence and hBD2 overexpression in human keratinocytes *PLoS One* 11(8): e0161227.

10. Fu Y, et al. (2005) Adiponectin promotes adipocyte differentiation, insulin sensitivity, and lipid accumulation *J Lipid Res* 46(7): 1369-1379.
11. Kim EJ, et al. (2016) UV-induced inhibition of adipokine production in subcutaneous fat aggravates dermal matrix degradation in human skin *Sci Rep* 6:25616.
12. Quan T, Fischer GJ (2015) Role of age-associated alterations of the dermal extracellular matrix microenvironment in human skin aging: a mini-review *Gerontology* 61(5): 427-434.
13. Shetty S, Kusminski CM, Scherer PE (2009) Adiponectin in health and disease: evaluation of adiponectin-targeted drug development strategies. *Trends Pharmacol Sci* 30(5): 234-239.
14. Yamauchi T, Kadowaki T (2008) Physiological and pathophysiological roles of adiponectin and adiponectin receptors in the integrated regulation of metabolic and cardiovascular diseases *Int J Obes (Lond)* 32: S13-18.
15. Choi JH, et al. (2010) Anti-diabetic drugs inhibit obesity-linked phosphorylation of PPARgamma by Cdk5 *Nature* 466: 451-456.
16. Kim MS, et al. (2005) The effects of a novel synthetic retinoid, Seletinoid G, on the expression of extracellular matrix proteins in aged human skin in vivo *Clin Chim Acta* 362(1-2): 161-169.
17. Rho HS, et al. (2014) Kojyl cinnamate ester derivatives promote adiponectin production during adipogenesis in human adipose tissue-derived mesenchymal stem cells *Bioorg Med Chem Lett* 24(9): 2141-2145.

17. Brun R.P, et al. (1996) Differential activation of adipogenesis by multiple PPAR isoforms. *Genes Dev.* 10(8): 974–984.
18. Evans RM, Barish GD, Wang YX (2004). PPARs and the complex journey to obesity *Nat Med* 10(4): 355-361.
19. Tontonoz P, Spiegelman BM (2008) Fat and beyond: the diverse biology of PPARgamma *Annu Rev Biochem* 77: 289-312.
20. Schmuth M, et al. (2014) Role of PPAR, LXR and PXR in epidermal homeostasis and inflammation *Biochim Biophys Acta* 1841(3): 463-473.
21. Ghadially R, et al. (1995) The aged epidermal permeability barrier. Structural, functional, and lipid biochemical abnormalities in humans and a senescent murine model *J Clin Invest* 95(5): 2281-2290.
22. Ezure T, Amano S (2007) Adiponectin and leptin up-regulate extracellular matrix production by dermal fibroblasts *Biofactors* 31(3-4): 229-236.
23. Akazawa Y, et al. (2011) Adiponectin resides in mouse skin and upregulates hyaluronan synthesis in dermal fibroblasts *Connective Tissue Res* 52(4): 322-328.
24. Tchkonja T, et al. (2010) Fat tissue, aging, and cellular senescence *Aging Cell* 9(5): 667-684.
25. Bluher M (2012) Clinical relevance of adipokines. *Diabetes Metab J* 36(5): 317-327.
26. Iwabu M, et al. (2015) Adiponectin/adiponectin receptor in disease and aging *npj Aging and Mechanisms of Disease* 1: 1-6.

27. Shibata S, et al. (2012) Adiponectin regulates cutaneous wound healing by promoting keratinocyte proliferation and migration via the ERK signaling pathway *J Immunol* 189(6): 3231-3141.
28. Sertznig P, et al. (2008) Peroxisome proliferator-activated receptors (PPARs) and the human skin *Am J Clin Dermatol* 9(1): 15-31.
29. Kuenzil S, Saurat JH (2003) Peroxisome proliferator-activated receptors in cutaneous biology *Br J Dermatol* 149(2): 229-236.
30. Icre G, et al. (2006) Functions of the peroxisome proliferator-activated receptor (PPAR)  $\alpha$  and  $\beta$  in skin homeostasis, epithelial reepair, and morphogenesis *J Invest Dermatol Symp Proc* 11(1): 30-35.
31. Sertznig P, Reichrath J (2011) Peroxisome proliferator-activated receptors (PPARs) in dermatology: the challenge and promise *Dermatoendocrinol* 3(3): 130-135.
32. Farmer SR (2006) Transcriptional control of adipocyte formation *Cell Metab* 4(4): 263-273.
33. Tsuchida A, et al. (2005) Peroxisome proliferator-activated receptor (PPAR) $\alpha$  activation increases adiponectin receptors and reduces obesity-related inflammation in adipose tissue *Diabetes* 54(12):3358-3370.
34. Goto T, et al. (2011) Activation of peroxisome proliferator-activated receptor-alpha stimulates both differentiation and fatty acid oxidation in adipocytes *J Lipid Res* 52(5): 873-884.

35. Hiuge A, et al. (2007) Effects of peroxisome proliferator-activated receptor ligands, bezafibrate and fenofibrate, on adiponectin level *Arterioscler Throm Vasc Biol* 27(9): 635-641.
36. Farmer SR (2005) Regulation of PPAR $\gamma$  activity during adipogenesis *Int J Obes (Lond)* 29 Suppl 1:S13-16.
37. Ahmadian M, et al. (2013) PPAR $\gamma$  signaling and metabolism: the good, the bad and the future *Nat Med* 19(5): 557-566.
38. Maeda N, et al. (2001) PPAR $\gamma$  ligands increase expression and plasma concentrations of adiponectin, an adipose-derived protein *Diabetes* 50(9): 2094-2099.
39. Caso G, et al. (2013) Peripheral fat loss and decline in adipogenesis in older humans *Metabolism* 62(3): 337-340.
40. Feingold KR., Schmuth M, Elias PM (2007) The regulation of permeability barrier homeostasis *J. Invest. Dermatol.* 127(7): 1574–1576.
41. Man, MQ, et al. (2006) Basis for improved permeability barrier homeostasis induced by PPAR and LXR activators: liposensors stimulate lipid synthesis, lamellar body secretion, and post-secondary lipid processing *J Invest Dermatol* 126(2): 386-392.
42. Feingold KR (2009) The outer frontier: the importance of lipid metabolism in the skin *J Lipid Res* 50 Suppl: S417-422.

43. Rivier M, et al. (2000) Peroxisome proliferator-activated receptor- $\alpha$  enhances lipid metabolism in a skin equivalent model *J Invest Dermatol* 114(4):681-687.
44. Ghadially R, et al. (1996) Decreased epidermal lipid synthesis accounts for altered barrier function in aged mice *J Invest Dermatol* 106(5): 1064-1069.
45. Harris IR, et al. (1997) Permeability barrier disruption coordinately regulates mRNA levels for key enzymes of cholesterol, fatty acid, and ceramide synthesis in the epidermis *J Invest Dermatol* 109(6): 783-787.
46. Jensen JM, et al. (2005) Acid and neutral sphingomyelinase, ceramide synthase, and acid ceramidase activities in cutaneous aging *Exp Dermatol* 14(8): 609-618.
47. Proksch E, Feingold KR, Elias PM (1992) Epidermal HMG CoA reductase activity in essential fatty acid deficiency: barrier requirements rather than eicosanoid generation regulate cholesterol synthesis *J Invest Dermatol* 99(2): 216-220.
48. Feingold KR, Jiang YJ (2011) The mechanisms by which lipids coordinately regulate the formation of the protein and lipid domains of the stratum corneum: Role of fatty acids, oxysterols, cholesterol sulfate and ceramides as signaling molecules *Dermatoendocrinol* 3(2): 113-118.
49. Mao-Qiang M, Feingold KR, Elias PM (1993). Inhibition of cholesterol and sphingolipid synthesis causes paradoxical effects on permeability barrier homeostasis *J Invest Dermatol* 101(2):185-190.
50. Feingold KR et al. (1990) Cholesterol synthesis is required for cutaneous barrier function in mice *J Clin Invest* 86(5): 1738-1745.



51. Chung JH, et al. (2007) Cholesterol inhibits MMP-9 expression in human epidermal keratinocytes and HaCaT cells *FEBS Lett.* 581(20): 3869-3874.
52. Kuhla A, et al. (2011) Aging is associated with a shift of fatty metabolism toward lipogenesis *J Gerontol A Biol Sci Med Sci* 66(11): 1192-1200.
53. Elias PM, et al. (1980) The permeability barrier in essential fatty acid deficiency: evidence for a direct role for linoleic acid in barrier function *J Invest Dermatol* 74(4): 230-233.
54. Sampath H, Ntambi JM (2011) The role of fatty acid desaturases in epidermal metabolism *Dermatoendocrinol* 3(2): 62-64.
55. Bolton-Smith C, Woodward M, Tavendale R (1997) .Evidence for age-related differences in the fatty acid composition of human adipose tissue, independent of diet *Eur J Clin Nutr* 51(9): 619-624.
56. Joo KM, et al. (2015) Relationship of ceramide-, and free fatty acid-cholesterol ratios in the stratum corneum with skin barrier function of normal, atopic dermatitis lesional and non-lesional skins *J Dermatol Sci* 77(1): 71-74.
57. Jungersted JM, et al. (2008) Lipids and skin barrier function--a clinical perspective *Contact Dermatitis* 58(5): 255-262.
58. Hanley K, et al. (1999) Fetal epidermal differentiation and barrier development *in vivo* is accelerated by nuclear hormone receptor activators *J Clin Invest* 113(5): 788-795.
59. Schmuth M, et al. (2004) Peroxisome proliferator-activated receptor (PPAR)-beta/delta stimulates differentiation and lipid accumulation in keratinocytes *J Invest Dermatol* 122(4): 971-983.

60. Wallmeyer L (2015) Stimulation of PPAR $\alpha$  normalizes the skin lipid ratio and improves the skin barrier of normal and filaggrin deficient reconstructed skin *J Dermatol Sci* 80(2): 102-110.
61. Hanley K, et al. (1998) Keratinocyte differentiation is stimulated by activators of the nuclear hormone receptor PPARalpha *J Invest Dermatol* 110(4): 368-375.
62. Hanley K, et al. (1997) Activators of the nuclear hormone receptors PPAR alpha and FXR accelerate the development the development of the fetal epidermal permeability barrier *J Clin Invest* 100(3): 705-712.
63. Demerjian M, et al. (2006) Topical treatment with thiazolidinediones, activators of peroxisome proliferator-activated receptor-gamma, normalizes epidermal homeostasis in a murine hyperproliferative disease model *Exp Dermatol* 15(3): 154-160.
64. Harris IR, et al. (1997) Permeability barrier disruption coordinately regulates mRNA levels for key enzymes of cholesterol, fatty acid, and ceramide synthesis in the epidermis *J Invest Dermatol* 109(6): 783-787.
65. Feingold KR (2012) Lamellar bodies: the key to cutaneous barrier function, *J. Invest. Dermatol.* 132(8): 1951–1953.
66. Schmuth M, et al. (2008) Thematic review series: skin lipids. Peroxisome proliferator-activated receptors and liver X receptors in epidermal biology *J Lipid Res* 49(3): 499-509.

67. Zettersten EM, et al. (1997) Optimal ratios of topical stratum corneum lipids improve barrier recovery in chronologically aged skin *J Am Acad Dermatol* 37(3 Pt 1): 403-408.
68. Hong I, et al. (2010) Activation of LXR $\alpha$  Induces Lipogenesis in HaCaT Cells *Arch Pharm Res* 33(9): 1443-1449.
69. Grubauer G, Feingold KR, Elias PM (1987) Relationship of epidermal lipogenesis to cutaneous barrier function *J Lipid Res* 28(6): 746-752.
70. Elias PM (2005) Stratum corneum defensive functions: an integrated view *J Invest Dermatol* 125(2): 183-200.
71. Elias PM, Ghadially R (2002) The aged epidermal permeability barrier: basis for functional abnormalities *Clin Geriatr Med* 18(1): 103-120.
72. Feingold KR, Elias PM (2014) Role of lipids in the formation and maintenance of the cutaneous permeability barrier *Biochim Biophys Acta* 1841(3): 280-294.
73. Kim EJ, et al. (2011) UV Modulation of Subcutaneous Fat Metabolism *J Invest Dermatol* 131(8): 1720-1726.
74. Shin MH, et al. (2016) Activation of peroxisome proliferator-activated receptor alpha improves aged and UV-irradiated skin by catalase induction *PLoS One* 11(9): e0162628.
75. Park MH, et al. (2013) The novel PPAR $\alpha/\gamma$  dual agonist MHY 966 modulates UVB-induced skin inflammation by inhibiting NF- $\kappa$ B activity *PLoS One* 8(10): e76820.

요약 (국문초록)

# 사람의 중간엽줄기세포에서 아디포넥틴 분비촉진 효과를 나타내는 코질신나메이트 유도체들의 타겟 규명 연구

김 세 언

서울대학교 약학대학원

제약학과 천연물과학 전공

노화에 따라 피부는 표피의 지질대사 기능의 저하로 피부장벽 기능이 약화되고, 피하지방층이 감소하여 주름 등의 표현형이 나타난다. 피부 항노화 물질을 발굴하기 위한 표현형 기반의 효능평가모델은 분자타겟 기반의 평가법에 비해 노화의 생리학적 조건과 유사한 환경에서 후보물질을 발굴하고 작용기전을 연구할 수 있다는 장점이 있다. 사람의 중간엽줄기세포는 지방세포, 골형성세포, 연골세포 등 다양한 세포로

분화하는 특징이 있어 항노화 물질 연구 및 다양한 질환의 신약 후보물질 탐색에 대표적인 세포표현형 모델로 활용된다. 본 연구에서는 사람의 지방조직유래 중간엽줄기세포의 지방분화 과정에 아디포넥틴 분비를 촉진하는 코질신나메이트(kojyl cinnamate) 유도체의 생리활성과 작용기전을 평가하였다. 코질신나메이트 유도체 중, 셀레티노이드 G 는 인체의 임상 실험에서 피부항노화 효과가 검증된 바 있지만, 분자약물학적 수준에서 항노화 작용기전은 불명확하다. 새로운 코질신나메이트 유도체들 중 사람의 중간엽줄기세포의 지방분화 과정에서 셀레티노이드 G 보다 아디포넥틴 분비촉진효과가 우수한 것을 확인하였다. 아디포넥틴 분비 촉진 효과가 우수한 코질신나메이트 유도체를 활용하여 분자약물학적 타겟을 규명하기 위해 지방조직의 분화 및 기능에 중요한 세포핵수용체인 퍼옥시솜 증식체-활성화 수용체(peroxisome proliferator-activated receptor, PPAR)에 대한 결합반응 실험을 수행하였다. 코질신나메이트 유도체들이 PPAR $\gamma$  와 PPAR $\alpha$  에 결합하였지만, PPAR $\delta$  에는 결합하지 않음을 확인하였다. 코질신나메이트 유도체들의 아디포넥틴 분비촉진 효과는 PPAR $\gamma$  와 PPAR $\alpha$  결합력과 상관성이 있음을 확인하였다. PPAR $\gamma$  와 PPAR $\alpha$  에 강한 결합력을 보이는 코질신나메이트 유도체는 지방조직유래 중간엽줄기세포와 각질형성세포의 콜레스테롤 대사에 중요한 HMGCR 과 HMGCS1 을 유의미하게 증가시켰다. 결론적으로 코질신나메이트 유도체들은 PPAR $\gamma$  와 PPAR $\alpha$  이중효능체로서 사람의 중간엽줄기세포의 아디포넥틴 분비를 촉진하고, 각질형성세포의

콜레스테롤 생합성 조절 효소의 발현을 증가시킴으로서 피부 항노화 효과를 나타낸다.

**주요어** : 표현형 기반 평가, 사람의 지방조직유래 중간엽줄기세포, 지방분화, 아디포넥틴, 퍼옥시솜 증식체-활성화 수용체

**학번** : 2015-22389